Evaluating Multimerization of Apoptin Homologs

A Major Qualifying Project

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by

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Abstract

Porcine Circovirus 1 Viral Protein 3 (PCV1 VP3), is homologous to Chicken Anemia Virus VP3 also known as Apoptin. Apoptin is known to induce apoptosis via a p53 independent pathway in cancerous cells and during this pathway Apoptin self-multimerizes. Due to the similarity of PCV1 VP3 to Apoptin, it is important to determine if multimerization is a crucial component in the apoptotic pathway. To investigate this, GFP and FLAG tagged VP3 constructs of PCV1 were transfected into non-small cell lung carcinoma H1299 cells. The VP3 interaction was then analyzed using co-immunoprecipitation and flow cytometry. Preliminary analysis showed that PCV1 VP3 does not multimerize, however further experiments are required to confirm this.

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Introduction

Cancer

Cancer is one of the leading causes of death across all age groups in humans. Recent statistics show that in one's lifetime, an individual has a 50% chance of developing cancer. Cancer is defined as the uncontrolled division of abnormal cells in a section of the body (American Cancer Society, 2014). It is generally caused by mutations in the cells which lead to the inhibition of cell safeguards such as p53. P53, a transcription factor associated with DNA repair, is vital in the up-regulation of cell cycle proteins, and stopping S phase during mitosis if a mutation in the genome is detected.

More than half of human cancer cells, however, do not possess the p53 gene and are able to bypass the cellular checkpoints in order to replicate. In other instances, there is a mutation of a cell's "self" marker; these markers or cell signals allow the body's immune system to identify cells as either part of the body or foreign. If cells are identified as intrusive, they are subsequently destroyed by lymphocytes. Cancer cells, however, are able to trick the immune system, remaining undetected by the the body are able to proliferate uncontrolled as natural killer cells have not yet evolved to recognize these markesr . From this growth, two types of tumors, or masses of cells, may form: benign and malignant.

Benign tumors are complexes of cells that closely resemble normal cells and are usually able to function as such. However, they are localized in the section of the body where the cells originated and do not invade other areas (National Institute of Health, 2013). In most cases, the tumor is able to be surgically removed and survival rate for patients is high. Conversely, malignant tumors are aggressive and able to spread. Malignant cancer cells contain mutated DNA and are considered immortal as they are able to turn off cell checkpoints, such as the p53

gene, that regulate cell growth (Voet et al., 2014). Without internal regulation, malignant cells are able to proliferate and, if located close to a blood vessel, travel throughout the body and spread to other areas. Some malignant cancer cells also possess high concentrations of survivin protein which inhibits apoptosis or programmed cell death. Other factors that contribute to growth of cancer cells include lack of contact inhibition and the ability to uncouple signal transduction stimulation from growth factor chemicals (Tamm et al., 1998).

Due to its prevalence in humans, treatments and cures for cancer are of high importance and interest to the medical community. Current treatments include the use of radiation and chemotherapy, however both lead to severe medical repercussions, most notably compromising of the immune system and the damaging of healthy cells. These treatments also focus on upregulating the p53 pathway, a natural tumor suppressor that is already present in human cells.

In recent years, a new promising form of cancer treatments has emerged: the use of oncolytic viruses. These treatments employ the use of nature's own agents to selectively detect and destroy malignant cells in the body. Examples of these viruses include avian adenovirus, non-human coronavirus, and yaba-like disease virus, all of which thrive in environments with quickly dividing cells (Vaha-Koskela et al., 2007). Another example of an oncolytic virus is Chicken Anemia Virus Apoptin. Apoptin is known to induce cell apoptosis in cancer cells independent of the p53 pathway as well as its homologs, human Torque Teno Virus and Porcine Circovirus.

Chicken Anemia Virus

Chicken Anemia Virus (CAV) is classified in the *Circoviridae* family and is the only member of the genus *Gyrovirus*. CAV was originally discovered in 1979 and described as an agent that was causing severe anemia in chicks. It was found to be a non-enveloped icosahedral

single stranded DNA virus that is ubiquitous in chickens and is distributed worldwide. The virus also works in tandem with other infections to suppress the immune systems and kill chicks less than a week old but is less infectious and fatal in chicks older than one week (Schat, 2009).

The CAV genome contains three overlapping Open Reading Frames, or ORFs. The first two reading frames, ORF 1 and ORF 2, are required for infection, structure, and replication of the virus within a host cell. ORF1 is believed to code for the capsid protein, the largest protein that is produced at approximately 51.6 kDa. ORF2 codes for a 25 kDa protein that facilitates replication and creation of the viral capsid. The third ORF codes for a 121 amino acid protein, viral protein 3, commonly referred to as Apoptin (Eisenberg et al., 2013). Apoptin is known to multimerize and induce apoptosis by interacting with and inhibiting, the Anaphase promoting complex/cyclosome (APC/C) in transformed cells, while leaving primary cells alone. (Heilman, 2006). CAV has several homologs that are believed to induce apoptosis in a similar manner. These homologs are present in humans, Torque Teno Virus, and in swine, Porcine Circovirus.

Torque Teno Virus

Torque Teno Virus (TTV) is currently classified in the *Circoviridae* family, genus Anellovirus and was first discovered in a non-A-E hepatitis patient in 1977 (Hino et al., 2007). It is a non-enveloped, single stranded circular DNA virus with a length of 3.8 kb that is highly stable and found in over 90% of humans. Although the virus was discovered almost forty years ago, little is known about its pathogenesis. Complications in gathering more information regarding the virus have risen due to its high genetic diversity and lack of good cell culture systems for viral propagation (Nishiyama, 2013). Regardless of the lack of information concerning the virus, 39 genotypes of TTV have been classified into 5 genogroups. Genotypes 1-4 are more prevalent and genotype five has not been fully established. Even with such high

variability there are three open reading frames within each strain that code for viral proteins 1, 2, and 3. Viral Protein 1 (VP1) facilitates the construction of the viral capsid; VP2 has been classified as a structural scaffold protein similar to the VP2 protein in Chicken Anemia Virus (Hino et al, 2007), and VP3 induces apoptosis in cancerous cells through a similar cellular pathway to CAV (Morassi, 2014).

Like Apoptin, TTV has been shown to induce apoptosis in oncogenic cells (Kooistra et al, 2004). Comparisons have also been drawn between TTV VP3 and CAV Apoptin's structures. Although little is known about TTV's function in the body, it has shown similarities to both parvoviruses and circoviruses, such as Porcine Circovirus.

Porcine Circovirus

Porcine Circovirus (PCV), discovered in 1974, is classified in the genus *Circovirus* of the *Circoviridae* family (Phenix et al, 2001) and was originally identified as a contaminant in porcine kidney cell cultures (Tischer et al, 1982). It is a non-enveloped, single stranded circular DNA virus with length of 1.76 kb and 17 nm in diameter (Phenix et al, 2001). Two genotypes of PCV have been discovered: PCV1 and PCV2. PCV1 is ubiquitous in swine but has not been found to cause any disease (Tischer et al, 1986). However, PCV2 has been found to be the leading cause involved in postweaning multisystemic wasting syndrome (PMWS) (Bolin et al, 2001). PCV2 is also further divided into two genotypes A and B, with B being much more virulent than A (Hanley, 2013).

PCV contains eleven identified open reading frames (ORFs) but only three are functional. These three ORFs produce four major proteins: Rep, Rep', Capsid and VP3. ORF1 produces the replicase protein (Rep) and its splicing variant Rep'. ORF2 produces the capsid protein (CAP). Finally ORF3 encodes for VP3, which is the protein associated with pathogenesis (Hanley,

2013). PCV1 and PCV2 have an overall DNA sequence homology of 68-78% with further analysis showing a 61.5% homology between their ORF3s (Liu et al., 2004). Alternatively, PCV2A and PCV2B have a 98% homology at the nucleotide level, with only 6 nucleotides or 3 amino acids that vary. The ORF3 or VP3 of PCV2 is similar to CAV VP3 and has been shown to exhibit similar apoptotic abilities (Hanley, 2013). Due to these similarities, it appears that analysis of PCV2 could be useful for cancer treatments.

Comparison of Homologs to Chicken Anemia Virus

TTV, CAV, and PCV1 and 2 show some structural and functional similarities, according to recent studies. The viruses are all classified within *Circoviridae* family and produce a third viral protein that is known to induce apoptosis. The sequences of the third open reading frames are homologues and the location of the nuclear export sequences (NES) and nuclear localization sequences (NLS) sequences align in the respective genomes. A visual representation of the third ORF of CAV Apoptin, PCV1, PCV2, and TTV can be seen in Figure 1 below.

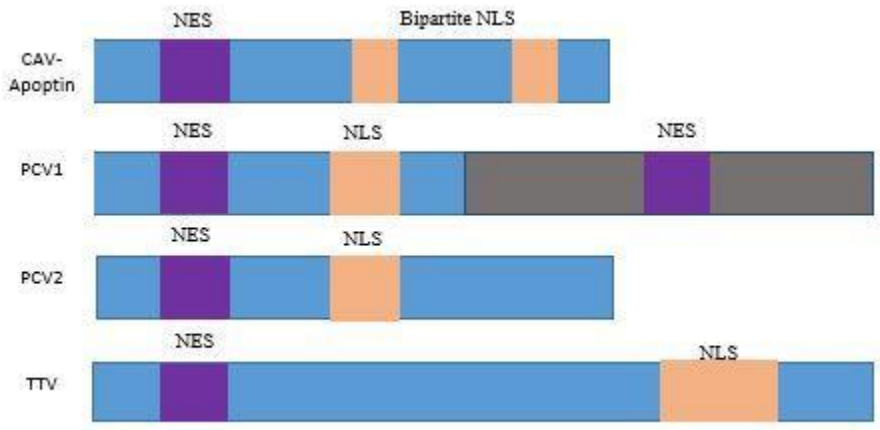

FIGURE 1:
SCHEMATIC OF LOCALIZATION AND EXPORT SEQUENCE OF CAV-APOPTIN AND ITS HOMOLOGS

From the structural similarities between the genomes it can be inferred that they behave and function similarity. It is known that Apoptin localizes in the nucleus, multimerizes, and triggers apoptosis. Studies have shown that Apoptin's nuclear localization sequence (NLS) and its nuclear export sequence (NES) are involved in inducing apoptosis. The belief is that movement of Apoptin into the nucleus signals the start of apoptosis (Heilman, 2006). Although structurally similar, Apoptin's homologs localize in cytoplasm. This potentially indicates that Apoptin's localization in the nucleus may or may not be as important as previously thought (Hanley, 2013).

TTV and CAV's open reading frames (ORFs) have high similarity with greater than an 80% match in a 36 nucleotide stretch near the replication origin. Also, they both appear to transcribe three or more spliced mRNAs. However, one puzzling factor is that the rest of the genome show no inherent similarity (Hino et al, 2008). In a recent study, TTV VP3 which included 105 amino acids from the source sequence TA278 was compared to Apoptin. It was determined that the apoptotic ability of TTV VP3, also known as TTV-derived apoptosis-inducing protein (TAIP), produced less of an effect than that of Apoptin (Kooistra, 2004). Although Apoptin and TTV VP3 localize in different parts of the cell, they both induce apoptosis independent of p53. Further investigation into their pathways can lead to a more definitive understanding of how these molecules are related.

PCV and its two isoforms, PCV1 and PCV2, have shown similarities both structurally and functionally to hTTV and CAV respectively. PCV1, like hTTV, appears to be nonpathogenic. While PCV2 is known to induce disease and immunosuppression similar to CAV, it also induces apoptosis in cancerous cells. Due to the high similarity between PCV, CAV, and hTTV investigation into their apoptotic pathways is warranted. Specifically whether

they multimerize to determine whether multimerization is an essential step in the apoptotic pathway.

Multimerization

Apoptin studies have demonstrated that when it enters the nucleus, it multimerizes and forms aggregates that contribute to the apoptosis of the cell. It has been found that the NES sequences and multimerization domains overlap (Heilman, 2006). The aggregates comprise about 30-40 subunits *in vitro*. It is suggested that the overall structure is spherical with beta-turns composing alternating hydrophobic and hydrophilic residues that maximize the interaction of the multimers with their surroundings (Leliveld, 2002).

Previous TTV VP3 studies have shown that a GFP tagged version of the viral protein forms aggregates in the cytoplasm of H1299 cancer cells. They do not penetrate the nuclear membrane even though they are located in proximity to it. They possess a sporadic shape, contrary to Apoptin which is inherently spherical. This sporadic shape could be due to weak protein interactions or an unknown secondary structure that produces the multimerization domain (Browning, 2009). Also in previous studies, PCV2 VP3 is believed to multimerize with itself and behave similarly to its homolog Apoptin (Liu et al., 2004).

It is important to determine whether TTV and PCV1 multimerize like Apoptin, which in turn may indicate whether or not multimerization is an important part of apoptosis. Finding other common features of these viral proteins can aid in the comprehension of the *Circoviridae* family and evaluate their probability as cancer treatments. This project will look into the multimerization ability of TTV VP3 and PCV1 VP3 and how it relates to that of Apoptin in order to get a better understanding of their apoptosis pathway.

Materials and Methods

Cell Culture

HI299 lung carcinoma cancer stock cells were suspended in D10 media comprised of DEMEM-HEPES, 10% fetal bovine serum, and 1% penicillin/streptomycin/fungicide in T-25 and T-75 tissue culture flasks and kept in an incubator at 37 degrees to optimize cell growth. After the cells had adhered to the flask and reached at least 75-80% confluence the cells were passed using the following procedure. The D10 media was aspirated off and the cells were washed with approximately 1 mL of PBS into the T-25 flask and 5 mL into the T-75. After curtaining the flask between 5-10 times, the PBS was aspirated off and the cells were trypsionized. For the T-25 1.5 mL and 3.0 mL in the T-75 of dilute (>.05%) trypsin protease solution was added to the flask and curtained. The excess trypsin was immediately aspirated out of the flask and the flask was incubated at room temperature for approximately a minute to allow the cells to detach from the flask. After this incubation, the cells were checked under a bright field microscope to determine whether the cells had detached. Once the cells had been lifted, 2 mL of fresh D10 media was added to the T-25 and 5 mL in the T-75 to remove the residual cells. The media was pipetted forcefully against the walls of the flask to break apart clumps of cells. 0.5 mL of the cells were transferred to a new T-25 flask along with 4 mL of D10 media. 2 mL of cells were transferred into a new T-75 flask and 18 mL of D10 media was added. Both flasks were checked under the microscope before being placed in the incubator to ensure enough cells had been passed. Cells that remained in the old flasks were aspirated and disposed.

Transfection

To analyze PCV1 VP3 in cancer cell conditions, the 3X FLAG and GFP PCV1 VP3 constructs were co-transfected into the H1299 cells, which are adherent non-small cell lung

carcinoma homozygous p53 null cells. Following the Effectene Transfection protocol for 6-well plates,4 µg total of the PCV1 constructs (2 µg per construct) were diluted into a final volume of 200µl of EC in 3 tubes (Apoptin FLAG/Apoptin GFP, PCV1 FLAG/PCV1 GFP, and No DNA control). From there, 6.4µl of enhancer was added to each tube, which was then vortexed and incubated for 2-5min at room temperature (RT). Then 20µl of effectene reagent was added to each tube and mixed by pipetting up and down. The complexes were allowed to form by incubating them at RT for 10-15 min. While the complexes are forming, the media was aspirated from the cells. The cells were then washed with 1x PBS and 1.5mL of new media was added to each well. After that, 1.2mL of media was added to the complexes and pipetted up and down to mix. Approximately, 710µl of this solution was then added dropwise to each of 2 wells. The dish was then gently swirled to evenly distribute the complexes in the dish. The cells were passed into dishes the day prior to transfection and then were transfected approximately 24 hours later when they had attained about 60-80% confluence.

The transfection efficiency was examined after 24 hours using a fluorescent microscope. Images from each well at 10X magnification are visible in Figure 3.

Cell Lysis and Immunoprecipitation

Approximately 24-48 hours post-transfection, the cells were harvested by aspirating the D10 media, washing with 1x PBS and leaving approximately 200µl in each well, scraping the cells with a cell scraper and resuspending them in the remaining PBS. The cell suspension was then transferred into a microcentrifuge tube and the wells that were the same constructs were combined. From there, the suspension was spun down for 5 min at 1500 rpm. After that, the cells were lysed by adding Buffer X to the cell suspensions and incubating them on ice for 20 min.

The cell extracts were then spun down at 9000 g for 30 min, following which, the supernatants was transferred to a new microcentrifuge tubes. 10µl of mouse EZview Red ANTI-FLAG M2 Affinity beads were then added to each of the new tubes containing the clarified cell extracts and then these were incubated end over end overnight at 4◦C. The following day, the samples were centrifuged at 1000 g for 60 seconds and then the supernatant was removed. Samples were then resuspended in 500µl of Buffer X. This washing step was repeated 5 times. After the fifth time, the supernatant was removed and the beads were resuspended in 50µl of 1x Gel Loading Buffer. To ensure denaturation of protein, the samples were then boiled for five min at 95◦C.

A full schematic of the experiments from transfection to flow cytometry is visible in Figure 2.

Flow Cytometry

Samples obtained from the immunoprecipitations were analyzed through flow cytometry using FL1-A gating for GFP and FL2-A gating for auto-fluorescence. After the cells had been resuspended in PBS, they were then transported on ice and covered in aluminum foil. An Accuri C6 Flow cytometer was used for analysis. A sample of distilled water was run through the machine for two minutes at a fast rate to clear any impurities or samples left from the previous run. The first sample was then loaded into the machine. The flow rate was set to 14 uL per minute for a total of 10,000 events at the slow rate.

Upon completion of this experiment, data obtained were analyzed using FlowJo computer software to generate dot plots and histograms. The original dot plots obtained from the data were set to have a y-axis of FSC-A or Forward Scatter and a x-axis of FL1-A, which is the channel where GFP is detected. These plots were then gated to isolate the main population without the

bead and cell debris. The plots generated from that gating were set to have a y-axis of FL1-A for GFP and a x-axis of FL2-A for auto-fluorescence. The population that appeared in the positive control but not the others was gated as the GFP positive population. Histograms were generated from the original dot plots to show the total GFP per sample with Count on the y-axis and FL1-A on the x-axis. Figures of the original dot plots, resulting dot plots and histograms can be seen in the Figure 4.

Results

As mentioned previously, cancer is generally caused by mutations in cells and in over half of cases, p53 and other cell checkpoints are not present. Because most cancers replicate without being checked, or stopped, by the p53 pathway, it is essential to investigate new treatments that also operate outside of this pathway. Apoptin is a viral protein known to induce apoptosis via a p53 independent pathway in cancerous cells and during this pathway, Apoptin self-multimerizes. Determining whether PCV1 possesses the ability to multimerize will aid in concluding if multimerization is important in the pathway to induce apoptosis in oncogenic cells. PCV1 VP3, an Apoptin homolog, was analyzed to determine if it also multimerizes in cancer cells, like Apoptin is known to do.

Proof of principle experiments were conducted to determine the best potential method of detection for VP3 multimerization. Immunoprecipitations are the traditional method for extracting a protein from a sample and were used for this investigation. Following immunoprecipitations, western blots, the accepted norm for protein detection from a sample, were performed. However, since western blots take a long time to perform and are expensive, a more efficient method was investigated. A sensitive alternative to western blotting is flow

cytometry, a useful analysis tool in many instances for protein quantification. Flow cytometry, contrary to western blots, is faster and more cost effective. Taking this into account, flow cytometry was chosen as a potential method.

To determine general multimerization capacity of Apoptin *in vitro*, GFP and FLAG tagged Apoptin constructs were co-transfected, as well as GFP and FLAG tagged PCV1 constructs, in human non-small cell lung carcinoma H1299 cells, along with no DNA control. Transfection of 2µg of DNA per well (1µg per construct), when looked at on the fluorescent microscope, yielded approximately 50-60% GFP expression, which can be seen in Figure 3. The co-transfected GFP and FLAG tagged Apoptin constructs were used to visually represent Apoptin's binding with itself. Assuming multimerization, in the case of the Apoptin constructs, co-transfection should yield co-expression and produce multimers that possess FLAG and GFP tags. Conversely, assuming no multimerization, in the case of GFP and FLAG Apoptin, co-transfection should yield co-expression that is later separated by FLAG-specific immunoprecipitation with EZ View ANTI- FLAG beads.

The samples were analyzed through flow cytometry using FL1-A gating for GFP and FL2-A gating for auto-fluorescence. The dot plots express where the scatter of beads reside in relation to the FL1-A channel on the Y axis and FL2-A channel on the X axis. These dot plots can be seen in row two of Figure 4. The FL2-A channel was chosen as a representation of auto-fluorescence due to the beads themselves, which are red in color, or cell debris released during the experiments. In the Apoptin panel, a distinct population is seen in the range of 10^{3} and 10^{4} that is not visible in the other two samples. This supports that Apoptin was the only one to multimerize. It is expected that Apoptin multimerizes, however the fact that it is shown in the dot plot confirms that the constructs work within this assay. When Apoptin multimerizes, the

aggregates formed are of very high molecular weight. According to the data, PCV1 does not multimerize. There are two reasons that could explain this; PCV1 does in fact not multimerize or the assay is not sensitive enough to detect multimerization at a lower magnitude. If PCV1 does multimerize, it may produce a multimer that is of a lower molecular weight than Apoptin, causing it not to be easily detected. The inhibition of forming such a large multimeric complex may be due to weak interaction between the proteins.

It can be seen on the dot plots that the populations are not very distinct. Because of this, histograms were produced from the dot plots, to get a better representation of the occurrence at each intensity for the FL1-A channel and can be seen in the third row of Figure 4. In the histograms, it is apparent that the population designated as GFP positive beads, from the Apoptin dot plot, is not present in the negative or PCV1 dot plots. The population is seen in the 10^{3} range. This provides even further evidence that Apoptin was the only one to multimerize.

The dot plots were also further analyzed to produce the percent frequencies of GFP and a bar graph was generated to allow for a more visual comparison between the samples. This bar graph can be seen in Figure 5. The first bar represents the percent frequency of GFP that appeared in the control sample, with a result of 0.044%. The second and largest bar correlates to the Apoptin sample with 50.1% frequency of GFP. Lastly, the third bar represents the PCV1 sample with 0.049% frequency. The discrepancy between the PCV1 and Apoptin samples on the graph provide further evidence to support that GFP Apoptin does multimerize while PCV1 does not.

Discussion

Although Porcine Circovirus Type 1 was discovered over 40 years ago, there are still many unanswered questions about how it functions. With it being easily able to infect porcine primary cells with no known adversative effects and able to selectively target and induce apoptosis in cancerous cells, it warrants further investigation into its capability as a cancer therapy.

The flow cytometry data showed a high level of scattering from the beads. Due to the non-uniformity between size and shape, there are large smears present in the flow data. Because immunoprecipitation beads were used, a non-traditional graph is produced when compared to the conventional live/dead cell model. However, this is inconsequential and does not impact the results that were obtained. Methods to prevent bead scatter could include disrupting the interaction between the FLAG tagged protein and the bead. Running just protein rather than proteins attached to beads through the flow cytometer, could potentially get rid of the scattering and produce a more traditional plot. It is unclear whether this will also disrupt the potential interaction between the GFP and FLAG tagged proteins, however this would be done at the end of the washes so any GFP tagged proteins present would remain in the sample. Because the GFP and FLAG tagged proteins may no longer be multimerized, it was suggested to increase the number of events from 10,000 in order to ensure that the GFP present in the sample gets run through the flow cytometer.

Another variable that was present in all the dot plots of the flow cytometry data was the presence of a second population of beads. The middle of the population is located at approximately 10^6 on the FL2-A axis in all the generated dot plots. The appearance of the population in all three samples suggests that it is caused by the bead themselves. There are

several ideas as to why there is a second population. Generally, flow cytometry is used to analyze live cells, specifically proteins that are fluorescing within one cell as it passes through a capillary, and usually those proteins that are not native to the cells themselves. Intensity in this case would be entirely dependent on the amount of protein that is giving off signal within the respective cell. However, this analysis was done using immunoprecipitation beads with potentially fluorescent proteins attached to them. The larger the clump of beads, the larger potential signal. The EZview Red ANTI-FLAG M2 Affinity beads that were used are composed of agarose, red dye, and ANTI-FLAG antibody. Due to their composition of agarose, the beads are known to cross-link which could cause the second population. Lastly, the beads are coated in ANTI-FLAG antibody producing multiple binding sites, which can potentially result in increased signal.

Finally, as previously stated, the dot plots and histograms show that, according to this assay, Apoptin was the only sample to multimerize. Flow cytometry is highly sensitive but that may also be one of its pitfalls. Apoptin's high molecular weight aggregates are an exaggerated example of multimerization. Apoptin studies show that its multimeric complex consists of approximately 30-40 monomers (Leliveld et al., 2003). The protein itself is already very large at 121 amino acids, therefore the complex is considerably large. The main discrepancy between the multimerization of Apoptin and the potential multimerization of PCV1 is the size of the multimer. PCV1 is not confirmed to multimerize and, therefore the size of its aggregates cannot be commented on. Even if PCV1 did multimerize, there is nothing known about the interaction, or strength of interaction, of the monomers produced. If the interactions are weak and small aggregates are formed, the assay that was used to detect large aggregates may not be sensitive enough to detect a signal. If the signal, or intensity, from the PCV1 is too low or not enough

monomers aggregate, it could be possible that the signal in the experiments conducted was lost in the portion gated out as cell debris.

Taking all of these variables into consideration, there is clearly a need to make this method more efficient for PCV1. One way to go about the size, or magnitude, discrepancy is to induce more crosslinking of the beads. Several ways of doing this are: adding a binding domain between the FLAG tagged constructs, adding a FLAG dimer to bridge beads, or adding Formaldehyde to induce crosslinking of proteins. If there is a low signal of multimerization of PCV1, these could be several ways to increase the magnitude of the signal for better detection.

Another thing to consider is the addition of the GFP tag to the Apoptin construct inhibits its apoptotic ability, while the apoptotic ability of the FLAG tagged Apoptin construct is uninhibited. However, the apoptotic ability, of both the GFP and FLAG tagged constructs of PCV1, is unaffected. Therefore, the apoptotic ability of the Apoptin constructs is only approximately half as strong as that of the PCV1 constructs. During experimentation, it was observed that 24 hours post-transfection, PCV1 had already begun to induce apoptosis as it was uninhibited in the transformed cells. Thus potentially reducing the GFP signal as after the cell is lysed the proteins begin to be degraded. It is also worth taking into consideration that, in general, PCV1 GFP fluoresces less brightly than Apoptin GFP.

Future Experiments

To further support or disprove the hypothesis that PCV1 does not multimerize, the experiments that were conducted should be optimized and repeated in triplicate. Flow cytometry is highly sensitive, but it may not be an optimal method for this assay. It has already been mentioned that the interaction between the PCV1 proteins, if any, may be too low to detect because the magnitude difference between the interaction of PCV1 proteins and the interaction

between the Apoptin proteins may be too great. Because of this, it may be necessary to prove, or disprove, that PCV1 multimerizes in the first place. This could be done with a western blot, where any potential signal could be detected.

Other variants of PCV such as PCV2 A and B should also be tested. Due to its homology with Apoptin and PCV and its known apoptotic abilities, production of the human Torque Teno Virus Viral Protein 3 Green Fluorescent Protein (hTTV VP3 GFP) construct is necessary in order for it to be studied as well. A design schematic for the creation of the hTTV VP3 GFP construct can be found in Figure 6.

Additionally, due to the red color of the EZview beads used, it is also highly recommended to use the standard non-colored ANTI-FLAG M2 Monoclonal Antibody Agarose Affinity Gel for the immunoprecipitations before flow cytometry analysis. Because these beads are also composed of agarose, a different type of bead could be used to decrease crosslinking between the beads.

As noted here, many other properties of the apoptotic process require further investigation. Examining the interactions between proteins within CAV, PCV, and TTV will allow for a better understanding of the mechanisms by which they achieve and induce cell apoptosis. Once this is understood, it will open a realm of possibilities for their use as cancer therapies.

Appendix

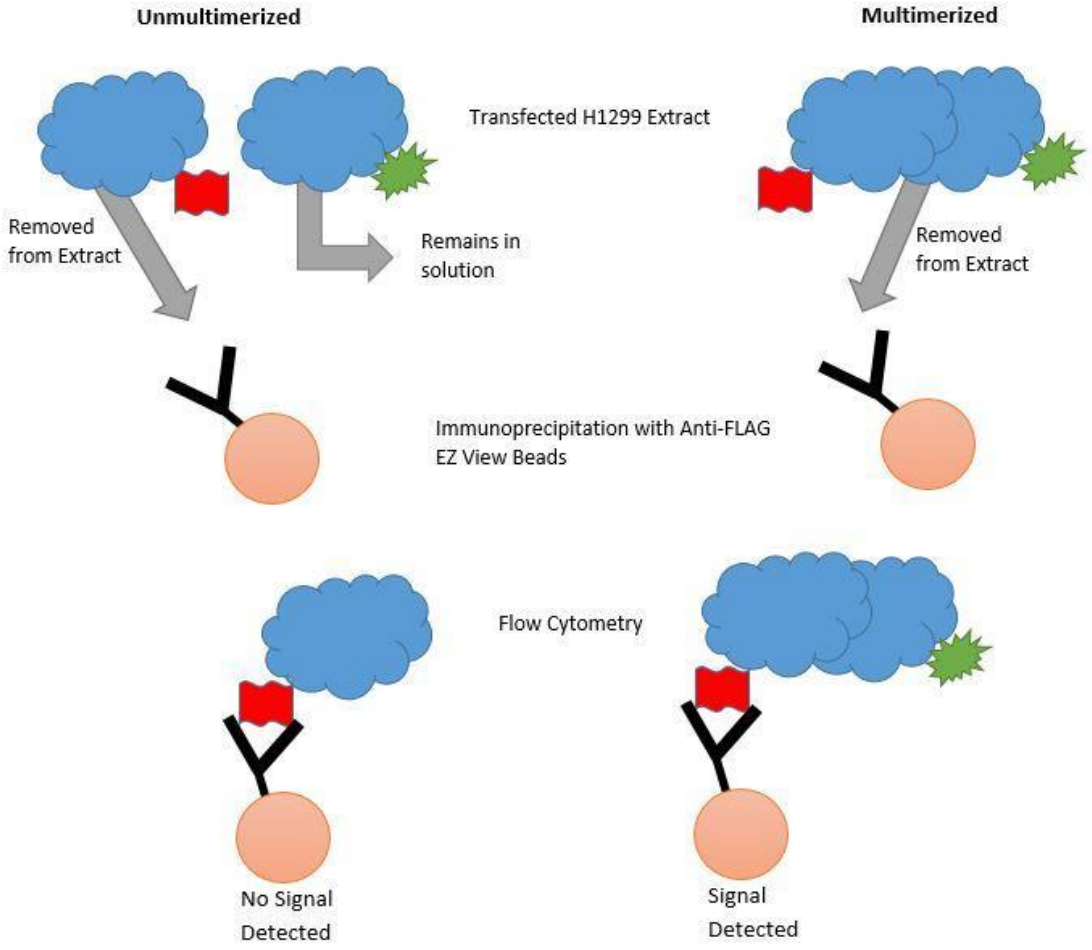

FIGURE 2: SCHEMATIC OF EXPERIMENTS CONDUCTED

To analyze the PCV1 and Apoptin fusion proteins under cancer cell conditions, 3X FLAG and GFP constructs were transfected into H1299 cells. Approximately 24-48 hours post-transfection images were taken under a fluorescent microscope to determine transfection efficiency. If the efficiency was greater than 60%, the cells were harvested and lysed. An immunoprecipitation was performed using EZview Red Anti-FLAG M2 Affinity beads before being analyzed through flow cytometry using the FL1-A gateway to screen for GFP. Figure 2 is a schematic of the experiments that were conducted to study the multimerization of Apoptin and PCV1.

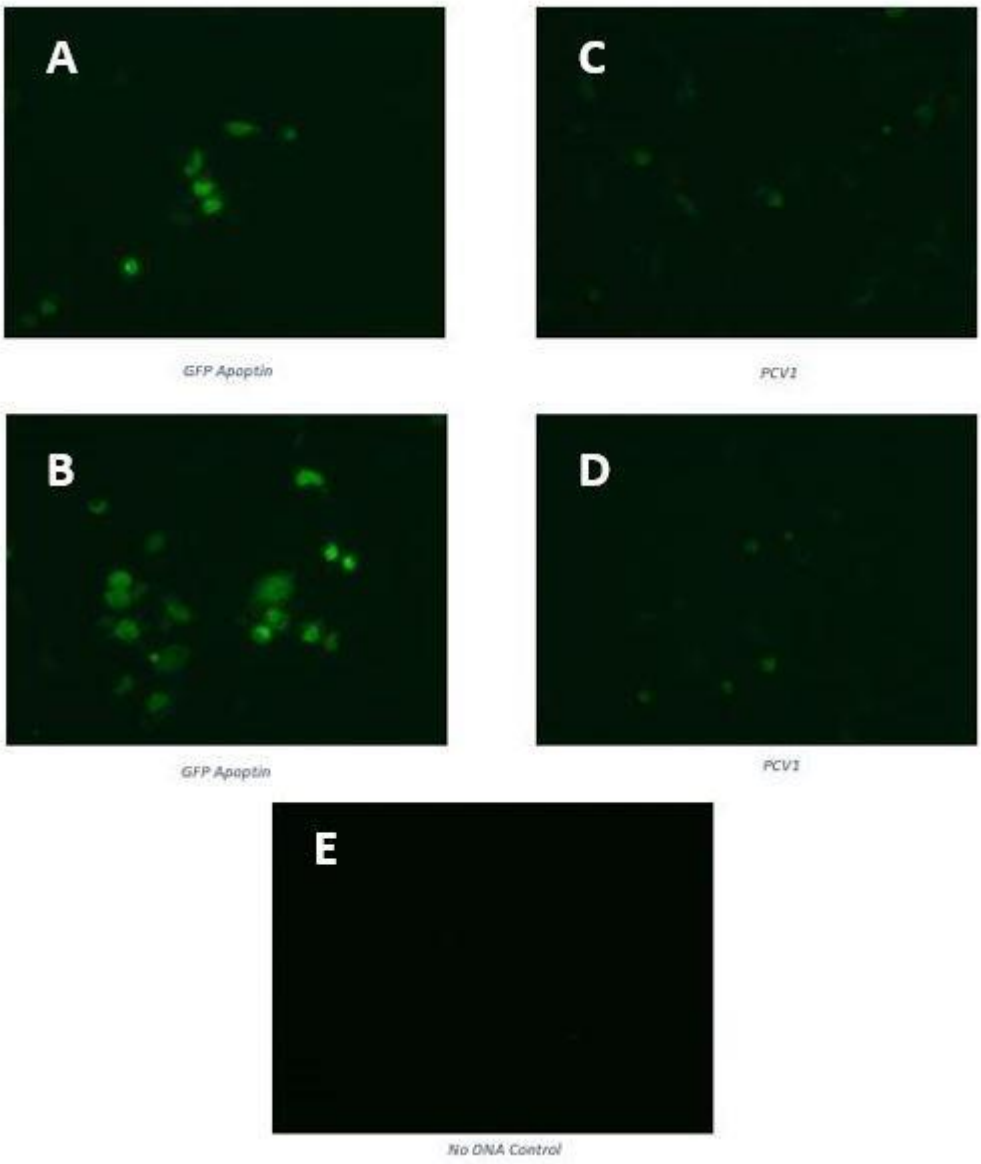

FIGURE 3: FLUORESCENCE MICROSCOPY IMAGES

Panels A and B represent GFP tagged Apoptin, Panels C and D represent GFP Tagged PCV1, and Panel E is the No DNA or negative control. All the constructs were transfected into H1299 cells and images were taken at 10X magnification 24 hours post-transfection. The GFP tagged Apoptin expressed the largest percentage of transfection with approximately 60% of the cells infected and expressing GFP. In contrast, PCV1 only had a 40-50% transfection efficiency and did not appear to fluoresce as brightly. The no DNA or negative control had no GFP expression and therefore appears completely black in the image.

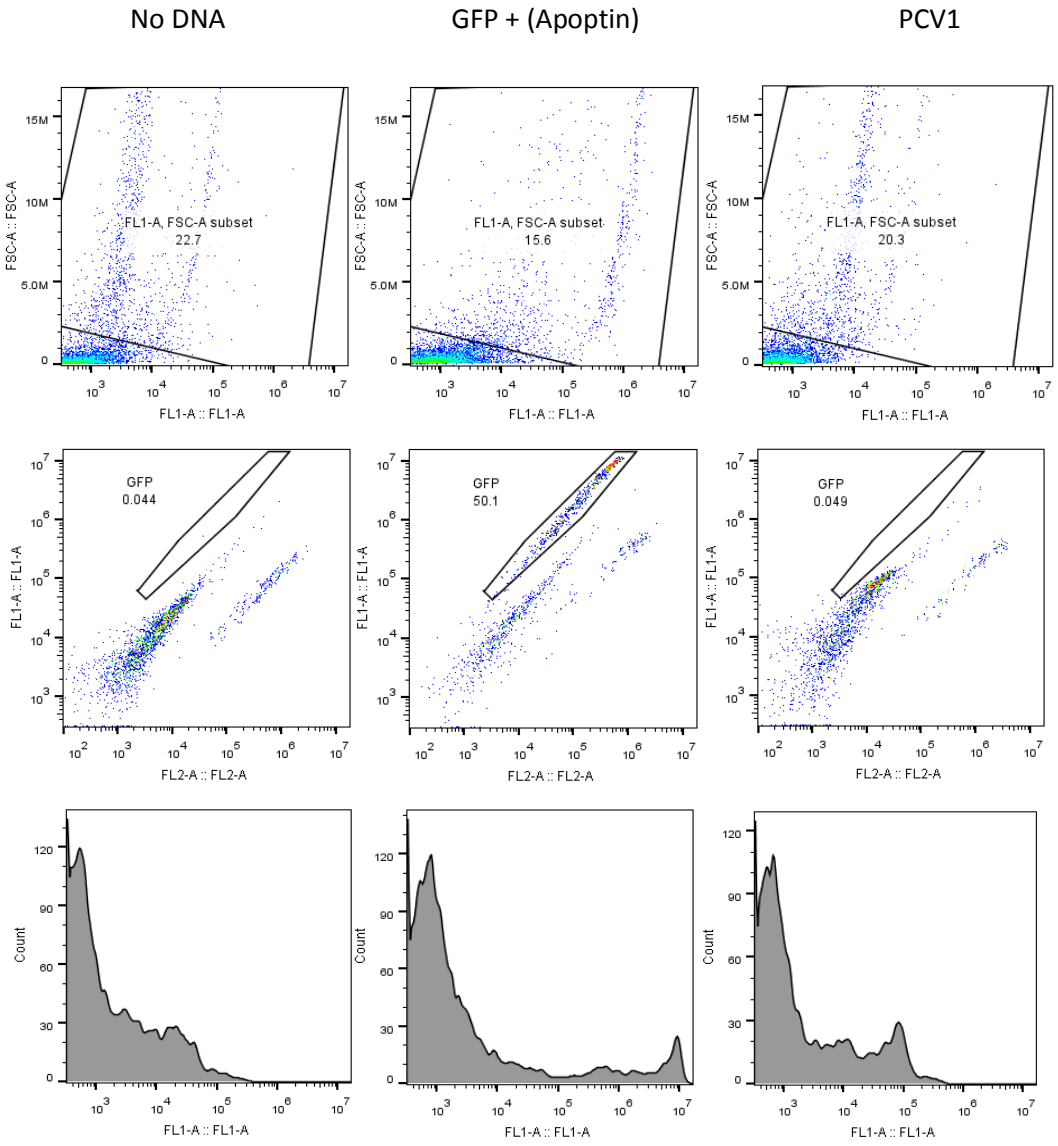

FIGURE 4: FLOW CYTOMETRY DOT PLOTS AND HISTOGRAMS

After transfection and imaging, an immunoprecipitation was performed and the samples were analyzed through flow cytometry using FL1-A gating for GFP and FL2-A gating for auto-fluorescence. The dot plots express where the scatter of beads reside in relation to the FL1-A channel on the Y axis and FL2-A channel on the X axis. FL2-A channel was chosen as a representation of auto-fluorescence due to the beads themselves or cell debris released during the experiments. In the GFP Apoptin panel, a distinct population is seen in the range of 10^5 and 10^7 and is not visible in the other two samples. From the dot plots, histograms were generated that measure bead count compared to the FL1-A gate.

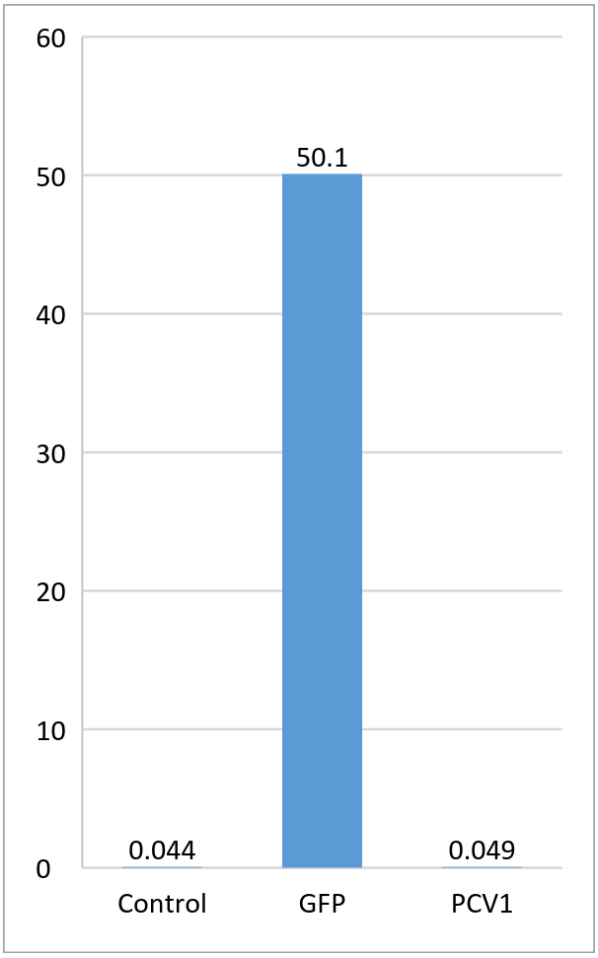

FIGURE 5: PERCENT FREQUENCIES OF GFP

The dot plots obtained from flow cytometry, as seen in Figure 4, were further analyzed to produce the percent frequencies of GFP and a bar graph was generated to allow for a more visual comparison between the samples. The first bar represents the percent frequency of GFP that appeared in the control sample, with a result of 0.044%. The second and largest bar correlates to the Apoptin sample with 50.1% frequency of GFP. Lastly, the third bar represents the PCV1 sample with 0.049% frequency.

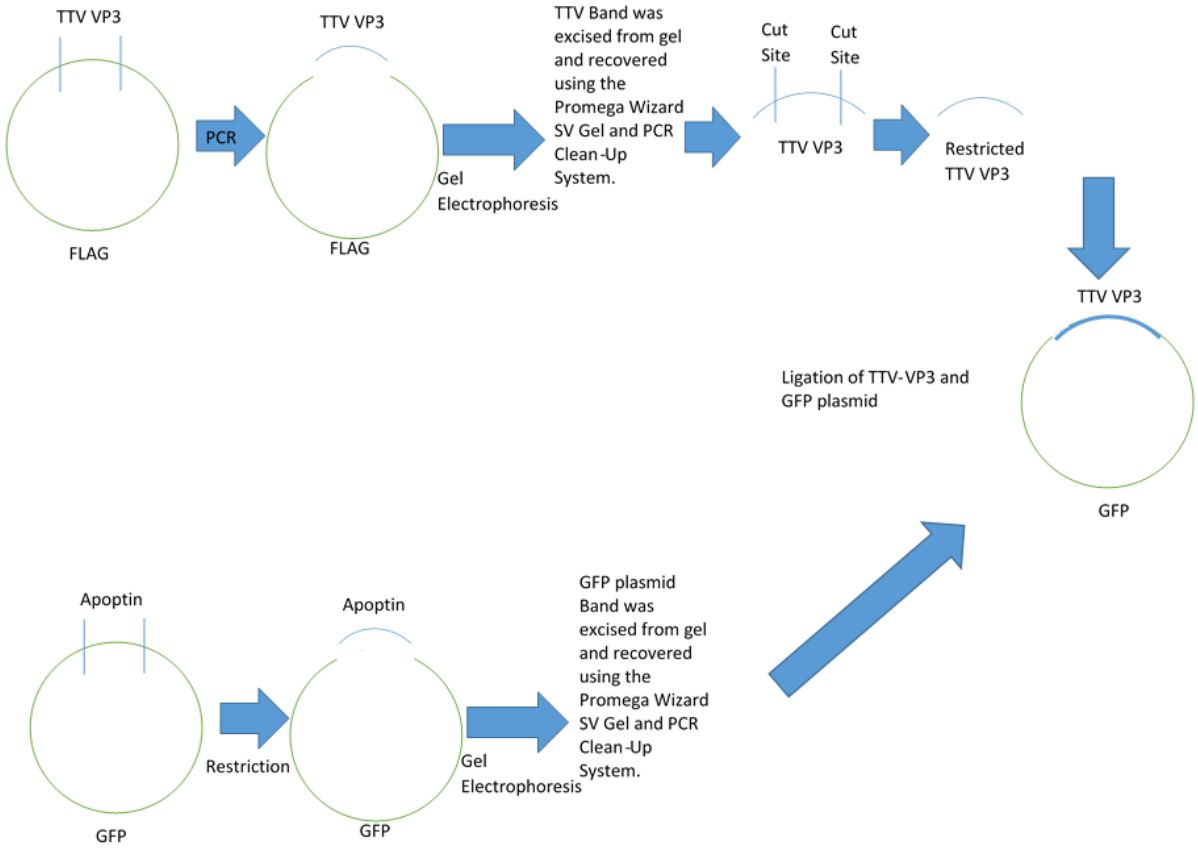

FIGURE 6: TTV VP3 GFP CONSTRUCT DESIGN SCHEMATIC

This schematic expresses the procedure that can be used to create the TTV VP3 GFP construct. TTV VP3 FLAG construct should be run through PCR to amplify the TTV VP3 region, then purified through gel electrophoresis. The TTV VP3 band should be excised, purified, then restricted. Apoptin GFP construct should be restricted to remove Apoptin and purified further to generate a GFP plasmid band. TTV VP3 and GFP plasmid are then ligated together to create the TTV VP3 GFP construct. Although, any construct containing GFP, not solely Apoptin GFP, can be used to create TTV VP3 GFP.

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